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Identification of the cysteine residue responsible for oxidative inactivation of mouse galectin-2

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Running title

The Cys residue mediating mGal-2 oxidative inactivation

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Abbreviations: BS^3, bis(sulfosuccinimidyl)suberate; CD, Circular dichroism; CysNO, S-nitrosocysteine; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline.

Summary

Galectins are a group of animal lectins characterized by their specificity for β-galactosides. Mouse galectin-2 (mGal-2) is predominantly expressed in the gastrointestinal tract and has been identified as one of the main gastric mucosal proteins that are uniquely sensitive to S-nitrosylation. We have previously reported that oxidation of mGal-2 by hydrogen peroxide (H_2O_2) resulted in the loss of sugar-binding ability, whereas pretreatment of mGal-2 with S-nitrosocysteine prevented H_2O_2-induced inactivation. In this study, we used point-mutated recombinant mGal-2 proteins to study which of the two highly conserved Cys residues in mGal-2 must be S-nitrosylated for protection against oxidative inactivation. Mutation of Cys^{57} to a Met residue (C57M) did not result in lectin inactivation following H_2O_2 treatment, whereas Cys^{75} mutation to Ser (C75S) led to significantly reduced lectin activity, as is the case for wild-type mGal-2. However, pretreatment of the C75S mutant with S-nitrosocysteine protected the protein from H_2O_2-induced inactivation. Therefore, Cys^{57} is suggested to be responsible for oxidative inactivation of the mGal-2 protein, and protection of the sulfhydryl group of the Cys^{57} in mGal-2 by S-nitrosylation is likely important for maintaining mGal-2 protein function in an oxidative environment such as the gastrointestinal tract.

Keywords: Galectin-2 / galectin / S-nitrosylation / oxidation / cysteine

Introduction

Galectins comprise a group of animal lectins that specifically bind to β-galactosides and are characterized by an evolutionarily conserved amino acid sequence motif in the carbohydrate-binding site (*1–3*). Galectins are known to be involved in a wide variety of biological processes including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and immune regulation (*4, 5*).

Among the galectins, galectin-2 (Gal-2) was first identified to be highly expressed in gastric cells, predominantly in epithelial cells of the rat stomach (*6*). Gastric mucous cells exhibit strong Gal-2 immunoreactivity, with expression observed in the small intestine (*7*); *GAL2* mRNA has also been detected in the human stomach (*8*). Taken together, the expression of Gal-2 in the gastrointestinal tract and the amelioration of acute and chronic colitis in mice by Gal-2 overexpression (*9*) as well as the reduced expression of human Gal-2 associated with lymph node metastasis in gastric cancer (*10*) and in *Helicobacter*-induced gastric cancer progression (*11*) have suggested that Gal-2 exerts a protective function in the gastrointestinal tract.

Gal-2 was identified in a screen of mouse gastric mucosal proteins that are uniquely sensitive to S-nitrosylation (*12*). This process, which involves the coupling of an NO group to the reactive thiol of a cysteine (Cys) residue in a polypeptide, is known to be an important post-translational modification in a variety of proteins (*13*). In addition to the NO generated enzymatically by NO synthase (NOS) from L-arginine by various cells including neurons and vascular endothelial cells, in which it functions as a signaling molecule, large quantities of NO are known to be generated in the stomach by non-enzymatic acid reduction of salivary nitrite to exert various physiological functions in the gastrointestinal tract (*14–17*) including as a barrier in the stomach (*18–20*). Several studies, including a study demonstrating that nitrate administration ameliorates dextran sulfate sodium-induced acute experimental colitis in mice (*21*), have suggested that the NO generated in the gastrointestinal tract might also have a protective role therein.

In contrast, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) can cause oxidative stress that could result in variety of disorders in the gastrointestinal tract including ulcers,

inflammatory bowel disease, and gastric and colorectal cancer (*22*). In our previous report (*23*), we found that although oxidation of mouse galectin-2 (mGal-2) by H_2O_2 resulted in the loss of sugar-binding activity, mGal-2 pretreatment with S-nitrosocysteine (CysNO) to effect S-nitrosylation prevented the H_2O_2-induced inactivation, presumably by protecting the Cys residues (Cys^{57} and Cys^{75}) in the protein that are highly conserved across mammals (*24*). Furthermore, analysis of mGal-2 proteins with site-directed mutations at the Cys^{57} and /or Cys^{75} positions suggested that both Cys residues were fully S-nitrosylated (*23*). In this report, we utilized wild-type and mutant forms of mGal-2 to investigate which of the two Cys residues in mGal-2 is responsible for its inactivation by H_2O_2-induced oxidation.

Materials and Methods

Expression and purification of recombinant mGal-2

The expression and purification of recombinant wild-type Gal-2 and the three mutated forms of mGal-2, C57M (Cys^{57} mutated to Met), C75S (Cys^{75} mutated to Ser), and C57M/C75S (Cys^{57} and Cys^{75} mutated to Met and Ser, respectively), were performed as previously described (*23*).

Measurement of protein concentration

Protein concentration was determined by Bradford's method (*25*) using bovine serum albumin as a standard.

Pretreatment reaction of recombinant mGal-2 proteins with CysNO for S-nitrosylation

The S-nitrosylation of the wild type and mutated forms of mGal-2 protein was performed as previously described (*23*). To remove unreacted CysNO, samples were ultrafiltered using Amicon Ultra centrifuge tubes (Millipore, Billerica, MA, USA).

Saville-Griess assay

The number of S-nitrosylated molecules was measured using the Saville-Griess assay as previously described (*23*, *26*).

Oxidation of mGal-2 by H_2O_2

The H_2O_2 concentration was measured at 240 nm (ε_{240} = 43.6 L/mol·cm). S-nitrosylated mGal-2 was incubated with 10 mM H_2O_2 for 2 h in the dark (*23*).

Binding of Gal-2 recombinant proteins to lactose after treatment with H_2O_2

C57M and C75S were treated with H_2O_2 and then subjected to a lactose-immobilized agarose (J-Oil Mills, Inc., Tokyo, Japan) column (bed volume 1 ml) as previously described (*23*). C75S was also pretreated with CysNO, followed by reaction with H_2O_2, and subjected to a lactose-immobilized agarose column as well.

Hemagglutination assay

The hemagglutination activity of the recombinant mGal-2 proteins was measured as previously reported (*23*, *27*). Briefly, a 25-μl sample of mGal-2 treated with CysNO and/or H_2O_2 was serially diluted 2-fold in a 96-well V-shaped microtiter plate, followed by the addition of 50 μl of EDTA-PBS and 25 μl of 4% (v/v) glutaraldehyde-fixed rabbit erythrocytes in EDTA-PBS. After the samples were allowed to stand for 1 h at room temperature, the end-point showing the minimum concentration resulting in hemagglutination was determined.

Circular dichroism (CD) spectra of recombinant mGal-2 mutants

The CD spectra of recombinant proteins at the concentration of 0.25 mg/ml in EDTA-PBS with or without 10 mM H_2O_2 were obtained using a J-720WI spectropolarimeter (JASCO Corporation, Tokyo, Japan).

SDS-PAGE under non-reducing and reducing conditions

The recombinant wild-type mGal-2 protein and its mutant forms, C57M, C75S, or C57M/C75S, were treated with or without H_2O_2 for 2 h in EDTA-PBS buffer. After the addition of catalase to quench the activity of the H_2O_2, sample buffer for SDS-PAGE with or without β-mercaptoethanol was added to the reaction mixture and the samples were incubated for 10 min at 70°C and subsequently subjected to SDS-PAGE. The gels were stained with Coomassie brilliant blue.

Crosslinking reaction using bis(sulfosuccinimidyl)suberate (BS^3)

The crosslinking reaction to detect multimer formation of the recombinant galectins using the BS^3 crosslinking reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) was performed as previously described (*23*), with some modifications. Briefly, after the S-nitrosylation reaction, recombinant mGal-2 (5 μg in 50 μL) was incubated with 0.3 mM BS^3 for 30 min in EDTA-PBS at room temperature. Samples were resolved by SDS-PAGE, and the gel was stained with Oriole stain (Bio-Rad).

Results

Binding capacity of recombinant mGal-2 to lactose

Recombinant wild-type mGal-2 was adsorbed to a lactose-immobilized agarose column and eluted with 0.1 M lactose. Oxidation of mGal-2 by H_2O_2 resulted in the loss of this binding ability and the recombinant mGal-2 ended up in the flow-through fraction, whereas pretreatment of mGal-2 with CysNO prior to the reaction with H_2O_2 prevented this binding inactivation and resulted in the generation of a lactose-eluted fraction (*23*). In this report, we used site-directed mutants of mGal-2, C57M, and C75S, and determined their susceptibility to H_2O_2-induced oxidation. To obtain the recombinant protein of the two mutants, C57M and C75S, recombinant proteins were expressed in *E. coli* and were subjected to a asialofetuin-immobilized agarose column with elution in 0.1 M lactose (data not shown) as described previously (*23*).

When recombinant C57M and C75S mGal-2 proteins were subjected to the lactose-immobilized agarose column after H_2O_2 treatment, only the C57M mGal-2 protein was adsorbed to the column and eluted with lactose (Fig. 1A). In contrast, as was the case for the wild-type mGal-2, the C75S mutant seemed to lose substantial ability to interact with immobilized lactose upon H_2O_2 treatment, and a large amount of the recombinant protein was observed in the flow-through fraction (Fig. 1B). However, when this C75S protein was pretreated with CysNO prior to the reaction with H_2O_2, the effect of H_2O_2 treatment disappeared, and the protein was adsorbed to the column and eluted with lactose (Fig. 1C); this outcome was the same as that observed for the wild-type mGal-2 treated with CysNO (*23*).

Hemagglutination activity

Similar to the results of the binding assay, the C57M mutant form of mGal-2 retained its hemagglutination activity after treatment with H_2O_2, whereas the wild-type form of mGal-2 lost this activity subsequent to H_2O_2-treatment (Fig. 2 line A (untreated) and 2B (H_2O_2-treated) wild-type mGal-2; Fig. 2D (untreated) and 2E (H_2O_2-treated) C57M). In contrast, treatment of the C75S mutant with H_2O_2 resulted in a 4-fold loss of hemagglutination activity (Fig. 2G, (untreated) and 2H (H_2O_2

treated) C75S). However, when C75S was incubated with CysNO prior to H_2O_2 treatment, the C75S mutant retained its hemagglutination activity (Fig. 2I), which was the same as the outcome for the wild-type protein (Fig. 2 C). For the Cys-less mutant C57M/C75S, the results were similar to those of the C57M mutant (Fig. 2J-L), in that no reduction in hemagglutination activity was observed even after H_2O_2 treatment.

CD spectrometry analysis of recombinant protein secondary structure

The CD spectra of the recombinant proteins dissolved in EDTA-PBS are shown in Fig. 3. Wild-type mGal-2 showed a typical β-sheet structure profile having a negative peak at the wavelength of 215 nm (Fig. 3A, thick bold line) resembling that of human galectin-1 (*28*); this peak was lost in the spectrum of H_2O_2-treated mGal-2 (Fig. 3A, thin line). When wild-type mGal-2 was incubated with CysNO prior to H_2O_2 treatment, the CD spectrum remained the same even after H_2O_2 treatment (Fig. 3A, dotted line). For C57M and the Cys-less form of mGal-2, in which Cys^{57} is mutated to a Met residue, the CD spectrum remained the same even after H_2O_2 treatment or incubation with CysNO (Fig. 3B (C57M), 3D (C57M/C75S)). However, when the C75S mutant, which retained the Cys^{57} residue in its polypeptide chain, was treated with H_2O_2, the change in the CD spectrum was the same as that observed for wild-type mGal-2; i.e., the typical peak resulting from the rich β-sheet structure was lost after H_2O_2 treatment but was retained when the C75S mutant was pretreated with CysNO prior to H_2O_2 treatment (Fig. 3C).

SDS-PAGE under reducing conditions

To test the formation of disulfide bonds between the Cys residues in the same polypeptide chain and/or two different polypeptide chains, recombinant proteins were treated with H_2O_2 and subjected to SDS-PAGE under reducing or non-reducing conditions; i.e., with or without β-mercaptoethanol in the sample buffer, respectively. Wild-type mGal-2 and the C57M, C75S, and C57M/C75S (Cys-less) forms of mGal-2 migrated to the position of a monomer at approximately 15 kDa under non-reducing conditions (Fig. 4A, lanes 1, 3, 5, and 7), and this result did not change after H_2O_2 treatment (Fig. 4A,

lanes 2, 4, 6, and 8). Similar results were obtained when the samples were treated with β-mercaptoethanol prior to SDS-PAGE (Fig. 4B), suggesting that no disulfide formation occurred after H_2O_2 treatment.

Detection of multimer formation of the wild-type and Cys-mutated mGal-2 proteins after H_2O_2 treatment by addition of the crosslinker BS^3

To test the change in multimerization of the recombinant proteins generated by H_2O_2 treatment, the recombinant proteins were incubated with the crosslinker BS^3 after H_2O_2 treatment, followed by SDS-PAGE and Oriole staining. When wild-type mGal-2 was incubated with BS^3, two primary bands were detected (Fig. 5, lane 1; the bands are indicated by single or double arrowheads). An additional band with a higher molecular weight was also observed after mGal-2 treatment with H_2O_2 (Fig. 5, lane 2; triple arrowheads). This additional band was not observed when the wild-type mGal-2 was pretreated with CysNO prior to H_2O_2 addition (Fig. 5, lane 3). The results for the C75S mutant, which still retains the intact Cys^{57}, were similar to those of the wild-type protein regarding the appearance of the higher-molecular-weight band (Fig. 5, lanes 7–9). However, when the two mutant proteins C57M and C57M/C75S, in which the Cys^{57} residue has been mutated to Met, were examined, the higher-molecular-weight bands were not observed after H_2O_2 treatment (Fig. 5, lanes 4–6 and 10–12).

Discussion

The polypeptide chain of mGal-2 contains two Cys residues within 130 amino acids; these two Cys residues are highly conserved among mammalian galectin-2 proteins. We have previously shown that mGal-2 loses its sugar-binding and hemagglutination ability by oxidative inactivation after treatment with H_2O_2 and that this inactivation can be prevented by pretreatment with CysNO (*23*). Furthermore, by using recombinant site-directed mutant proteins of mGal-2, i.e., C57M, C75S, and C57M/C75S, our results suggest that the two Cys residues, Cys^{57} and Cys^{75}, are both S-nitrosylated by treatment with CysNO (*23*).

The Cys^{57} residue was changed to Met instead of Ser because the C57S mutant had low solubility as previously reported (*23, 29*). Because the two mutants, C57M and C75S, were both adsorbed onto the asialofetuin-agarose column and could be eluted specifically with 0.1 M lactose, with an elution profile similar to that of wild-type mGal-2 (data not shown), the amino acid substitutions did not affect the binding activity of the two mutants for β-galactoside-containing sugar structures. We also tried to express the recombinant C57A protein of mGal-2 (Cys^{57} residue changed to Ala) to eliminate any potential effects of the modification of the Met residue. However, this mutant also had low solubility and it was difficult to obtain sufficient amounts of the recombinant protein, as previously reported (*23, 29*).

When the ability of the recombinant proteins to be adsorbed to β-galactosides was tested after H_2O_2-treatment, the C57M mutant retained its ability to bind to the β-galactoside-immobilized column and was eluted specifically with lactose. In contrast, for the C75S mutant treated with H_2O_2, a substantial amount of the protein was found in the flow-through fraction, indicating that oxidative inactivation caused the loss of the sugar-binding ability of the protein. The finding that some remaining proteins were still adsorbed to the column likely resulted from the residual recombinant proteins not being fully inactivated because of the high concentration of the recombinant protein in the solution. However, when the C75S mutant was pretreated with CysNO prior to exposure of the protein to H_2O_2, the loss of the sugar-binding ability was abrogated, similar to the outcome observed for the wild-type protein as reported in our previous study (*23*). Furthermore, when the hemagglutination activities of these mutants were tested, the C75S mutant showed a substantial loss of hemagglutination following exposure to H_2O_2, whereas pretreatment with CysNO abrogated this loss of function, again as observed for wild-type mGal-2. In contrast, neither the sugar-binding nor the hemagglutination ability of C57M or C57M/C75S was reduced by H_2O_2 treatment. Because the wild-type and C75S mutant, which retained an intact Cys^{57} residue, were susceptible to H_2O_2-induced oxidative inactivation, whereas the C57M and C57M/C75S mutants, which did not have the –SH group of the Cys^{57} residue, were not, these results suggest that the Cys^{57} residue in the mGal-2 protein is responsible for the H_2O_2-induced oxidative inactivation of mGal-2. Furthermore, protecting this

Cys^{57} by CysNO treatment appears to be critical for protecting the lectin ability of mGal-2 from oxidative inactivation.

Galectins, including Gal-2, have a typical structure that is rich in β-strands (*30*) and have a typical β-sheet profile, with a negative peak on CD analysis at 215 nm as shown in Fig 3A for wild-type mGal-2 (solid line). When the lectin is treated with H_2O_2, this typical negative peak disappears, indicating that the secondary structure of the lectin has been lost. However, mutants C57M and C57M/C75S, whose Cys^{57} is mutated to Met and which do not have the –SH at this position, maintained their basic spectra even after H_2O_2 treatment. Furthermore, when the wild-type protein and C75S mutant, which retained the –SH side chain at the Cys^{57} position, were pretreated with CysNO prior to H_2O_2 exposure, they maintained their CD spectrum with the typical negative peak for the β-strands even after H_2O_2 treatment. These results suggest that the secondary structure remained intact, possibly as a result of S-nitrosylation of the Cys^{57} residue. Therefore, we concluded that the oxidation of the Cys^{57} residue by H_2O_2 treatment induces degeneration of the lectin structure and results in loss of lectin activities such as β-galactoside binding and erythrocyte hemagglutination, and that protection of the –SH group on the Cys^{57} residue by CysNO pretreatment retains their activity under oxidative inactivating conditions.

The SDS-PAGE result shown in Fig. 4 indicates that H_2O_2 treatment does not induce the formation of disulfide bridges between the two mGal-2 molecules. A very faint band that corresponds to the position of a possible dimer was observed in the case of the C75S mutant after H_2O_2-treatment; this band probably resulted from the boiling in the sample buffer without β-ME prior to SDS-PAGE. Furthermore, from the result of the cross-linking experiment, the denaturation of the mGal-2 protein by H_2O_2 treatment might have exposed the hydrophobic region originally folded inside the structure, thus resulting in a multimeric aggregation product. In fact, after treatment with H_2O_2, an insoluble precipitant was observed in the tube (not shown).

According to the X-ray crystallographic structure of human Gal-2 in complex with lactose (*30*), the Cys^{75} residue exists on the other side of the mGal-2 molecule from the surface involved in dimer formation. Therefore, it is highly unlikely that the two Cys^{75} residues in the mGal-2 homodimer form

a disulfide bond in an oxidative environment. However, very small amount of artificial dimer generated by the disulfide bond formation between the two Cys^{75} residues from two independent mGal-2 molecules, resulting in another very faint band for the H_2O_2-treated C57M mutant of mGal-2 (Fig. 4A, lane 4), may underlie the stronger hemagglutination activity observed for the wild-type protein and C57M mutant treated with H_2O_2 (Fig. 4, lines C, E, and F. For line B, the wild-type mGal-2 is inactivated by oxidation via Cys^{57}.) The C57M/C75S mutant, which does not have Cys^{75}, did not show this stronger hemagglutination activity after H_2O_2 treatment (Fig. 4, lines K and L), which could support this idea.

Gal-1 is known to form intramolecular disulfide bonds (*31*) and to have different biological functions depending on its redox status (*28, 32–35*). However, Gal-1 has also been shown to be inactivated by oxidation using H_2O_2, and redox regulation has been reported to be involved in the structure-function relationship of Gal-1 (*28*). Furthermore, oxidation of the chicken galectin CG-1B (C14) leads to two forms of homodimers through formation of either an intrasubunit disulfide bond between Cys^2 and Cys^7 or an intersubunit disulfide bond between the two Cys^7 (*36*). Thus, the function of Gal-2 in the stomach might also be regulated by its redox status, and S-nitrosylation could play an important role in this regulation. However, although the loss of lectin activity of Gal-1 is reported to result from formation of intramolecular disulfide bonds, this cannot be the case for the oxidative inactivation of mGal-2 because the C75S mutant, which only has one Cys residue remaining at the Cys^{57} position, is also susceptible to inactivation by H_2O_2 treatment.

Protein tyrosine phosphatases (PTPs) are reported to be reversibly oxidized and inactivated when cells are treated with H_2O_2, and they are thus suggested to be regulated by oxidization (*37*). PTP1B, an enzyme of the PTP family that is important for negative regulation of the insulin receptor (*38,39*), is oxidized by H_2O_2 at the essential residue Cys^{215} (*40*). This Cys^{215} residue has been proposed to be reversibly inactivated to sulfenic acid (*40*), and it has also been reported that this Cys residue could be irreversibly oxidized to sulfinic or sulfonic acid states (*41*) and that S-nitrosylation of Cys^{215} protected PTP1B from oxidation-induced inactivation (*42*). X-ray crystallographic analysis of PTP1B showed

that, during reversible inactivation, Cys^{215} was oxidized to sulfenic acid and then to a sulphenyl-amide structure, which forced a conformational change in the main chain of the protein (*43,44*).

Cys^{57} is located in the middle of the carbohydrate-binding cassette with its side chain sulfhydryl group protruding to the side opposite that of the β-sheet that binds lactose. A conformational change similar to that in PTP1B (*43, 44*) induced by oxidation of the Cys^{57} residue in mGal-2 could be the cause of the loss of the binding ability of mGal-2 for the β-galactoside-containing column and also the loss of the hemagglutination activity of this protein. However, considering that the typical peak resulting from the rich β-sheet structure was lost in the CD spectra of both the wild-type and C75S proteins when treated with H_2O_2, and that a higher-molecular-weight complex was formed as shown in Fig. 5, some portion of this protein could be irreversibly oxidized to the sulfinic or sulfonic acid states at the Cys^{57} residue, resulting in permanent degeneration of the whole structure. Although it remains unclear whether the oxidation at this position is reversible at the sulfenic acid level or irreversible at the sulfinic or sulfonic acid level, protection of the sulfhydryl group of Cys^{57} by S-nitrosylation should be beneficial for maintaining lectin activity under oxidative stress, as there were no substantial differences in the sugar-binding profile or hemagglutination activity of mGal-2 with and without CysNO treatment (*23*).

It has been reported that Gal-1 is also S-nitrosylated at Cys^{43} and Cys^{61} (*45*); notably, Cys^{61} is located at the position corresponding to Cys^{57} of Gal-2. Additional experiments to determine whether or not Gal-1 is also protected from oxidative inactivation by S-nitrosylation are therefore warranted.

In conclusion, our study demonstrated that Cys^{57} is likely to be responsible for oxidative inactivation of the mGal-2 protein and that protection of the sulfhydryl group of the Cys^{57} in mGal-2 by S-nitrosylation might be important for maintaining mGal-2 protein function in an oxidative environment such as the gastrointestinal tract. The strong immunoreactivity observed in gastric mucous cells and the suggestion that Gal-2 might have a protective function in the gastrointestinal tract indicates that Gal-2 might have a role similar to that of Gal-3, which has been reported to play an important role in maintaining mucosal barrier function through carbohydrate-dependent interactions with cell surface mucins (*46, 47*). Furthermore, because the NO generated in the gastrointestinal tract

has been suggested to have a protective role therein, and no substantial changes in the carbohydrate binding activity have been detected between the S-nitrosylated and non-S-nitrosylated forms of Gal-2, it is conceivable that the S-nitrosylation of Cys^{57} in Gal-2 might be one of the main factors maintaining the interactions of Gal-2 with carbohydrate-containing molecules in the stomach mucus such as mucins, resulting in a more stable mucosal barrier function. To build on these results, more detailed analyses of the molecular mechanisms underlying oxidative inactivation and the means by which this inactivation is prevented by CysNO pretreatment are necessary and ongoing in our laboratory.

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Figure legends

Fig. 1 Effect of H_2O_2 treatment on the sugar-binding profiles of recombinant mGal-2 C57M, C75S, and C75S pretreated with CysNO.

Purified recombinant mGal-2 mutants (C57M and C75S) were treated with 10 mM H_2O_2 for 2 h in the dark, added to a lactose-immobilized agarose column, washed with EDTA-PBS, and eluted with lactose. The elution profiles of mGal-2 were verified by measuring the protein concentration of each fraction (0.5 ml each) in addition to SDS-PAGE analysis. Bands at approximately 15 kDa, the molecular weight of the mGal-2 monomer, were detected by Coomassie brilliant blue staining. Profiles of recombinant mGal-2 C57M (**A**), C75S (**B**), and mGal-2 C75S pretreated with CysNO (CysNO+) before H_2O_2 treatment (**C**) as eluted from the lactose-immobilized agarose.

Fig. 2 Effect of H_2O_2 treatment on the hemagglutination of mGal-2 mutants pretreated with CysNO.

mGal-2 mutants were treated with 10 mM H_2O_2 for 2 h in the dark and the treated recombinant protein (250 μg/mL) was diluted 2-fold in a 96-well microtiter plate. A suspension of glutaraldehyde-fixed rabbit erythrocytes in EDTA-PBS was added and the hemagglutination activity was determined after a 1 h of incubation. Lanes A, B, and C, wild-type mGal-2; lanes D, E, and F, C57M mutant; lanes G, H, and I, C75S mutant; lanes J, K, and L, C57M/C75S mutant.

Fig. 3 Effect of H_2O_2 treatment on the secondary structure of mGal-2 mutants pretreated with CysNO.

The CD spectra of recombinant mGal-2 mutants, recombinant proteins treated with 10 mM H_2O_2 for 2 h in the dark, and recombinant proteins pretreated with CysNO and treated with H_2O_2 are shown for (**A**) wild-type, (**B**) C57M, (**C**) C75S, and (**D**) C57M/C75S.

Fig. 4 SDS-PAGE analysis of H_2O_2-treated mGal-2 mutants under non-reducing and reducing conditions.

Recombinant mGal-2 was treated with 10 mM H_2O_2 for 2 h in the dark and was subjected to SDS-PAGE under non-reducing (**A**) and reducing (**B**) conditions.

Fig. 5 Detection of multimer formation of H_2O_2-treated mGal-2 using the crosslinker BS^3.

BS^3 was added to a recombinant mGal-2 solution with (CysNO+) or without (CysNO−) pretreatment prior to addition of H_2O_2 (H_2O_2+). The reaction mixture was subjected to SDS-PAGE and the gel was stained with Oriole. The results for wild-type and C57M mutant proteins are shown in (**A**), and those for the C75S and C57M/C75S mutants are shown in (**B**). The positions of the Gal-2 monomer, dimer, and multimer are shown by one, two, or three arrowheads, respectively. The bands for catalase, which is added to remove the remaining H_2O_2 activity, are also indicated.

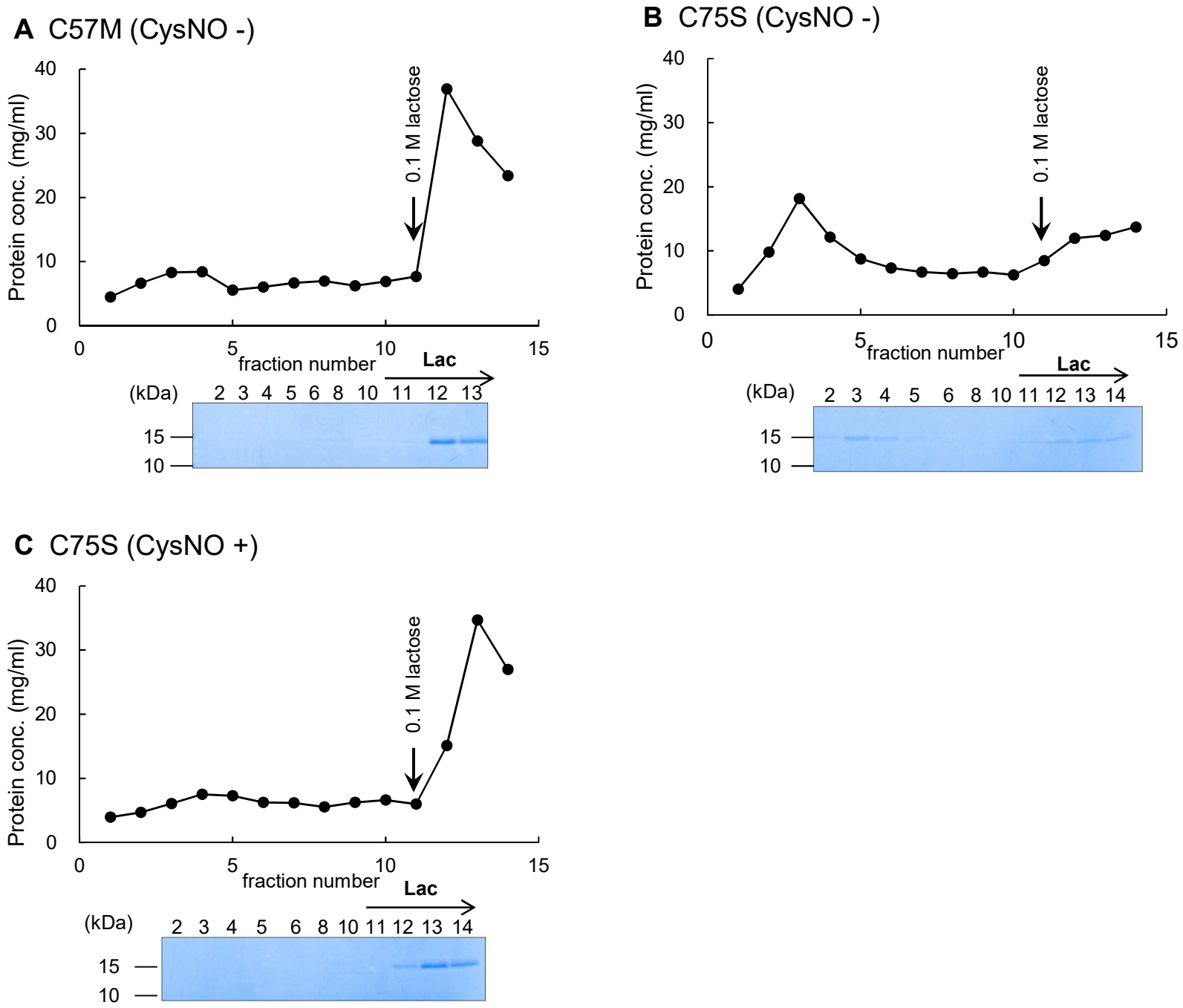
A C57M (CysNO -)
Protein conc. (mg/ml)
0.1 M lactose
fraction number
Lac
(kDa)
2 3 4 5 6 8 10 11 12 13
15
10
B C75S (CysNO -)
Protein conc. (mg/ml)
0.1 M lactose
fraction number
Lac
(kDa)
2 3 4 5 6 8 10 11 12 13 14
15
10
C C75S (CysNO +)
Protein conc. (mg/ml)
0.1 M lactose
fraction number
Lac
(kDa)
2 3 4 5 6 8 10 11 12 13 14
15
10

Fig. 1

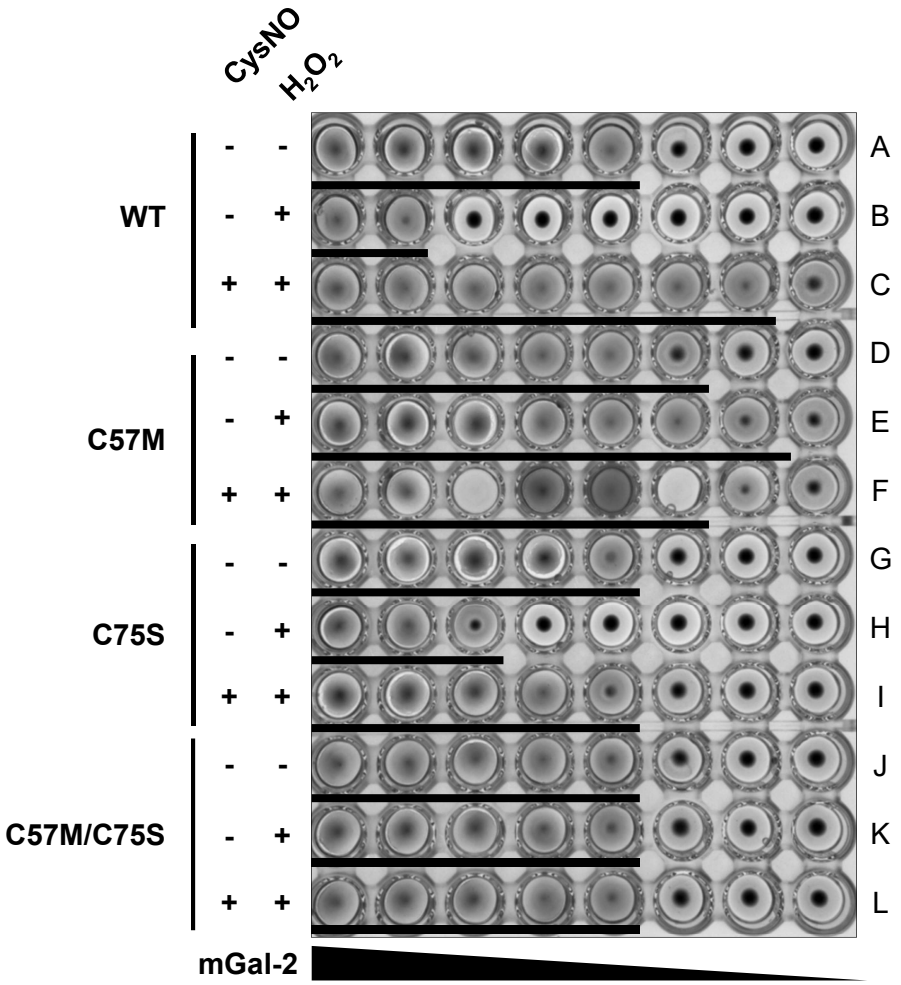

Fig. 2

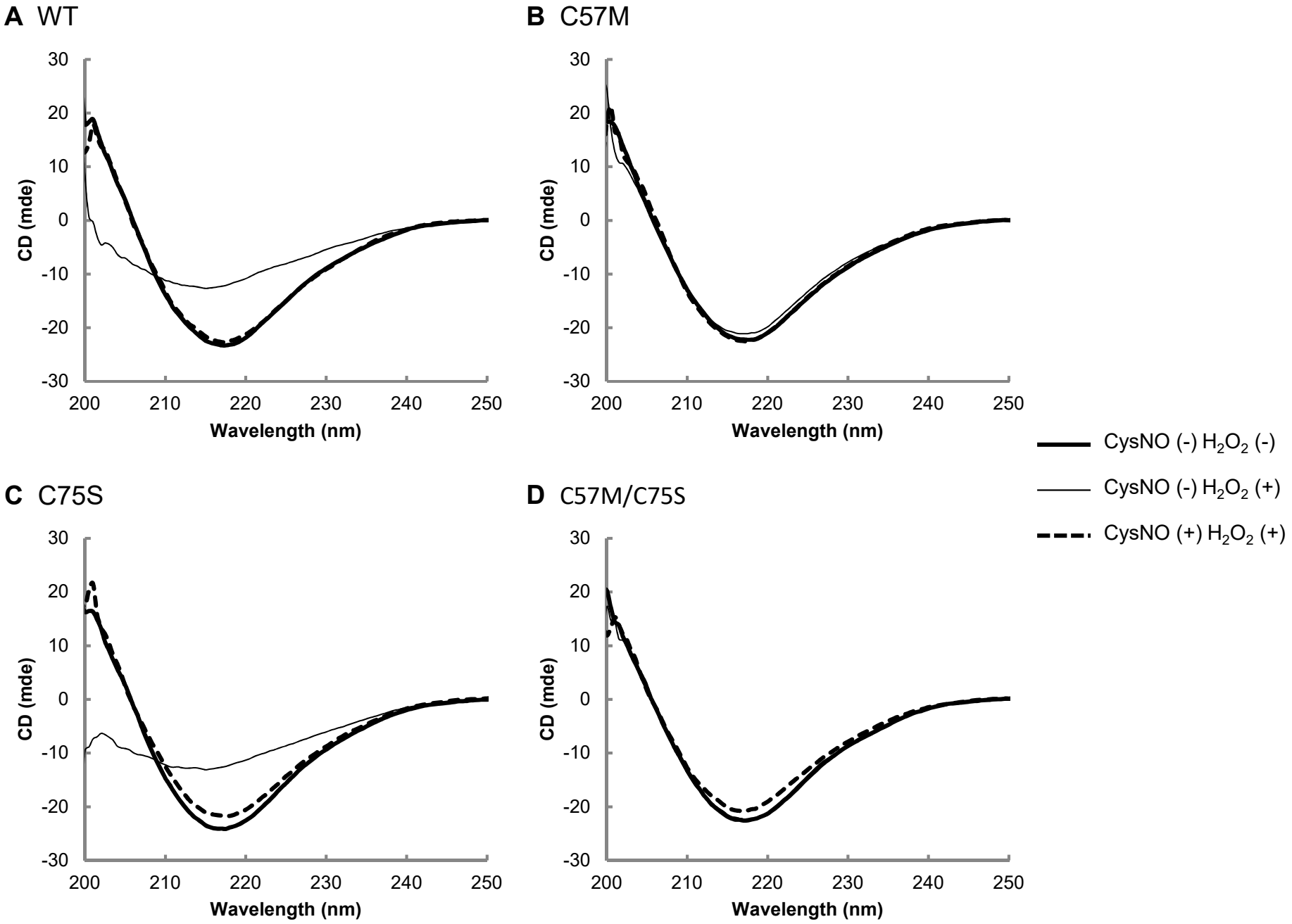
A WT
B C57M
C C75S
D C57M/C75S
CD (mde)
Wavelength (nm)
30
20
10
0
-10
-20
-30
200
210
220
230
240
250
CysNO (-) H2O2 (-)
CysNO (-) H2O2 (+)
CysNO (+) H2O2 (+)

Fig. 3

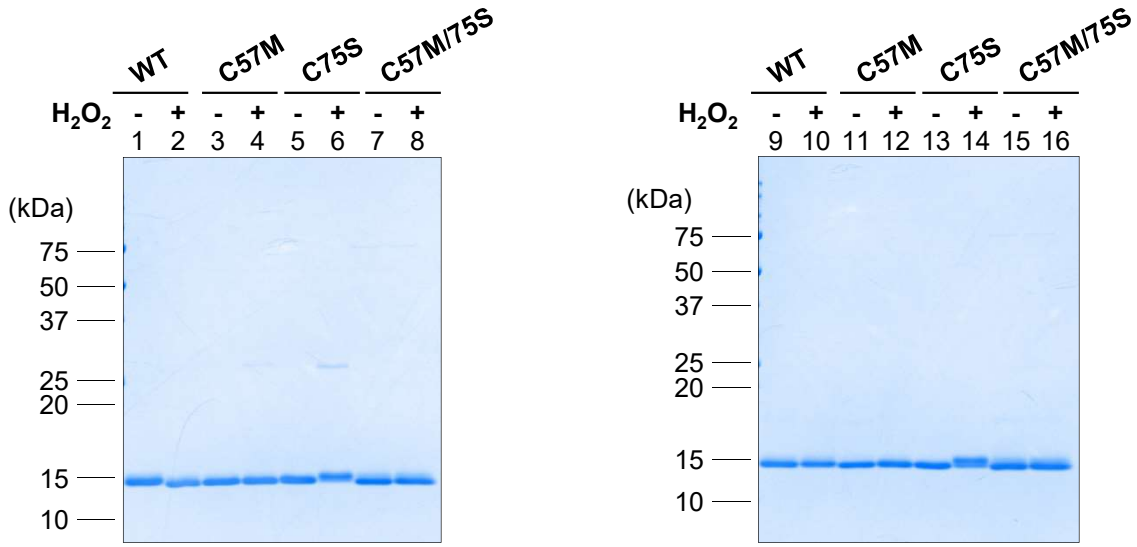
A Non-reducing
B Reducing
WT
C57M
C75S
C57M/75S
H2O2
- + - + - + - +
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
(kDa)
75
50
37
25
20
15
10

Fig. 4

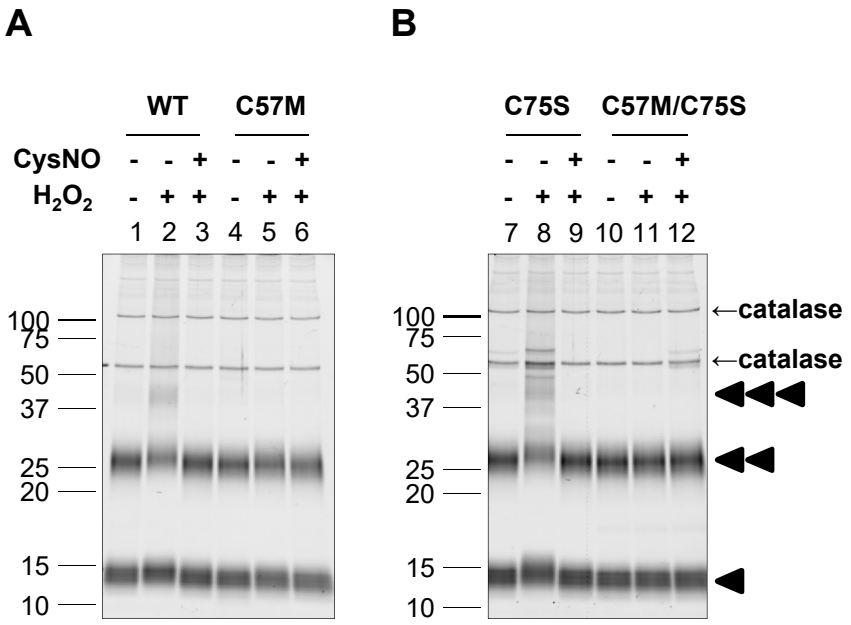
A
B
WT
C57M
C75S
C57M/C75S
CysNO
H2O2
1 2 3 4 5 6
7 8 9 10 11 12
100
75
50
37
25
20
15
10
←catalase
←catalase

Fig. 5